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Defective Fin Regeneration in Medaka Fish (*Oryzias latipes*) with Hypothyroidism

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Wild-type medaka are known to have remarkable capabilities of fin, or epimorphic, regeneration. However, a hypothyroid mutant, *kamaitachi* (*kmi*), frequently suffers from injury in fins, suggesting an important role of thyroid hormone in fin regeneration. This led us to examine the relationship between thyroid hormone and fin regeneration using medaka as a model. For this, we first set up a medaka experimental system in which the rate of regeneration was statistically analyzed after caudal fin amputation under normal and hypothyroid conditions. As expected, the regeneration of amputated caudal fins was delayed in hypothyroid *kmi* –/– mutants. We then examined wild-type medaka with thiourea-induced hypothyroidism to evaluate the requirement of thyroid hormone during epimorphic fin regeneration. The results demonstrate that the growth rate of regenerates was much reduced in severely hypothyroid medaka throughout the regeneration period. This reduction in regenerative rate was recovered by exogenous administration of L-thyroxine. The present study is thus the first to report the direct involvement of thyroid hormone in teleost fin regeneration, and provides a basic framework for future molecular and genetic analyses.

Key words: thyroid hormone, hypothyroidism, fin regeneration, epimorphic regeneration, medaka, teleost

INTRODUCTION

Urodele amphibians and teleost fish are known to possess remarkable capabilities of epimorphic regeneration. In particular, they can fully regenerate their severed appendages. In striking contrast, regenerative abilities of mammalian limbs are extremely limited. Studies on organ regeneration have been conducted mainly in amphibians (Brockes and Kumar, 2002), but recently, small teleost fish such as zebrafish and medaka have attracted much research in this field (Akimenko *et al*., 2003; Poss *et al*., 2003; Katogi *et al*., 2004). Compared with urodele amphibians, teleost fish have several advantages: (1) their fins are relatively simple, but still complex enough to dissect the processes of organ regeneration; (2) molecular tools and massive genome sequences are available; (3) fin amputation is easy and a non-lethal surgery; (4) the regeneration process is easy to observe from outside as well as under the microscope through transparent fins; (5) the fish can be maintained at a large scale; (6) they are amenable to genetic analyses, because fish regeneration mutants are beginning to emerge (Nechiporuk *et al*., 2003; Poss *et al*., 2002; Makino *et al*., 2005; Whitehead *et al*., 2005). Furthermore, a large-scale analysis of the genes involved in fin regeneration was conducted in medaka by Katogi *et al.* (2004)

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The key observation leading to the present study was the phenotype of a medaka mutant. Since medaka have a strong capability of fin regeneration, one rarely sees a healthy adult medaka with damaged fins in laboratory aquaria. However, we frequently observed that the medaka mutant *kamaitachi* (*kmi*) suffered from injury in fins. *kmi* is a medaka spontaneous mutant that lacks immunoreactivity with thyroxine (T4) in the thyroid follicle. This suggested an important role of thyroid hormone in fin regeneration.

Thyroid hormone is a biologically important regulator for metabolism, cell proliferation, apoptosis, differentiation, and metamorphosis (Hadley, 2000). It acts on nearly all tissues through thyroid hormone nuclear receptors, and thus the biological function of thyroid hormone depends on the cellular context of a target tissue. In organ regeneration, for example, thyroid hormone is known to promote the proliferation of hepatocytes after partial hepatectomy (Malik *et al*., 2003; Moro *et al*., 2004). In contrast, our knowledge of the role of thyroid hormone in epimorphic regeneration has been limited, and there are only a few reports in lizards that hypothyroidism inhibits epimorphic tail regeneration (Turner and Tipton, 1971; Ramachandran *et al*., 1996). Studies with lizards, however, face difficulties in the molecular dissection of the regeneration process, because of limited availability of genetic and genomic resources. We thus used the medaka as a model to obtain molecular insights into the mode of action of thyroid hormone in epimorphic regeneration.

In this study, as a first step, we set up a medaka experimental system with which to analyze the role of

thyroid hormone in fin regeneration. We found that the regeneration of amputated caudal fins is delayed in hypothyroid *kmi* mutants. We then examined wild-type medaka with pharmacologically induced hypothyroidism to evaluate the requirement of thyroid hormone during epimorphic fin regeneration. These results provide a basic framework for future molecular and genetic analyses.

MATERIALS AND METHODS

Animals

All experiments were performed using adult medaka fish (*Oryzias latipes*) derived from southern Japanese wild-type populations. All adult fish we used were sexually mature and laid eggs constantly. All females and males were paired, and each pair was maintained in a 2-liter plastic aquarium at 27°C on a lighting regime of 14 hr light and 10 hr dark. Water was exchanged daily.

Immunohistochemistry

Whole-mount antibody staining was performed as described previously (Elsalini and Rohr, 2003; Alt *et al*., 2006). A polyclonal anti-thyroxine antibody (anti-T4, 1:4000 rabbit anti-thyroxine BSA serum; ICN Biomedicals, #65-850) and a polyclonal anti-human thyroglobulin antibody (anti-TG, 1:4000 rabbit anti-Human thyroglobulin; Dako, #A0251) were used to visualize the thyroid follicle in medaka. For staining, hatching-stage larvae were used. Fertilized eggs were collected and incubated at 30°C. Medaka larvae usually hatch around 8 days post-fertilization. Larvae were fixed in PFA at 4°C for 12 hr, rinsed in phosphate-buffered saline with 0.1% Tween 20 (PBT), and stored in methanol at –20°C. In order to block endogenous peroxidases, embryos were incubated in 2-ml tubes with 1.5 ml of 10% H_2O_2 in methanol for 12 hr, then 1 ml of the solution was exchanged with fresh PBT, mixed, and incubated for a further12–16 hr. Larvae were then rinsed in PBT, blocked in 1% blocking reagent (Roche, #1096176) for 2 hr, incubated with anti-T4 or anti-TG for 2 hr, and rinsed in PBT for 2 hr. The larvae were then incubated with biotinylated anti-rabbit IgG (1:500; Vector, #BA100) for 2 hr, rinsed in PBT for 2 hr, and incubated with an ABC Kit solution (Vector #PK6101) for 2 hr. Finally, they were rinsed again in PBT for 2 hr and once in PBS, and incubated in DAB (0.2 mg/ml PBS) for 10 min. For staining, 1 μl of 0.3% H_2O_2 solution was added. To stop the reaction, larvae were rinsed once in PBT and postfixed in PFA for 15 min, after which they were rinsed in PBT and stored in 70% glycerol. All procedures were carried out at room temperature.

Fin amputation and measurement of regenerative length

For amputation experiments, 3 mm of the ventral half of the caudal fins of adult fish were amputated with a razor blade (see Fig. 4 A, B). Digital images of regenerates were taken, and rays 2 and 3 (see Fig. 4A) were measured using ImageJ software (http://rsb.info.nih.gov/ij/), and the average distance between the two rays was recorded. The tissues of hypothyroid regenerates were so weak that they often became damaged. In the case of missing or shortened rays 2 or/and 3, rays 1 or 4 were measured instead. These rays were chosen because, when amputated, they usually regenerate to the same length.

Pharmacological treatments

To obtain fish with hypothyoroidism, thiourea (Wako, #204-01202) treatment was performed as described previously (Tagawa and Hirano, 1991). For this, each pair was reared in 2 liters of fresh water containing 0.003% thiourea. For T4 replacement experiments, L-T4 (30 ng/ml; MP Biomedicals, Inc. #152145) was administered in addition to thiourea. The water with chemicals was changed daily.

Plasma sampling and radioimmunoassay

Plasma samples were collected directly from the caudal artery of the fish using a micro capillary, as described previously (Iwata *et al*., 1982). Total T4 and 3,5,3'-triiodothyronin (T3) concentrations in plasma were measured by specific radioimmunoassays as described previously (Tagawa and Hirano, 1987, 1989).

Statistical analysis

All statistical data were expressed as means, with vertical bars representing standard errors of the means. For parametric distributions, the *F*-test was applied to estimate differences in variances. When there were no statistical differences among variances ($P>0.05$), Student's *t*-test was applied to compare means. When there were significant differences among variances ($P<0.05$), Welch's *t*-test was applied. For nonparametric distributions, the Mann-Whitney *U*-test was applied.

RESULTS AND DISCUSSION

kamaitachi mutant

Kamaitachi (kmi) is a recessive spontaneous mutant that was isolated from the orange-red variety of a medaka southern population in our recent screening, which focused on thyroid development. In the screening, we used an antibody against L-thyroxine that visualizes the lumen of larval thyroid follicles (Elsalini and Rohr, 2003; also see Fig. 1A), and we isolated the *kmi* mutant, which is devoid of thyroid hormone immunoreactivity (Fig. 1B). Surprisingly, *kmi* mutants are homo viable and able to mature sexually, which enabled us to maintain this strain in a homozygous state. Thus in the following experiments, we used *kmi* mutant fish derived from homozygous parents. Based on these observations, we reasoned that *kmi* mutants would display mild hypothyroidism with defects in the storage of thyroid hormone and/or efficient hormone production.

To further characterize *kmi* mutants, we used an antibody for human thyroglobulin, a precursor of T4 (anti-TG), which was reported to stain zebrafish thyroid follicles (Alt *et al*., 2006). Although the epitope of the anti-TG is not defined, this antibody likely recognizes TG independent of thyroglobulin iodination, because in zebrafish treatment with goitrogen suppresses T4 immunostaining but does not affect TG immunostaining (Alt *et al*., -2006). Like zebrafish, the pattern of TG immunostaining in wild-type medaka (Fig. 1C) resembles that of T4 immunostaining (Fig. 1A). When *kmi* larvae were stained with anti-TG antibody, weak but numerous signals were detected (Fig. 1D), indicating that mutant follicle cells are capable of producing TG. However, the gross morphology of the mutant follicles was not normal; compared with wild-type follicles, the stained thyroid follicles of *kmi* were irregular in shape and smaller in size (Fig. 1D). These results strongly suggest that *kmi* mutants manage to develop differentiated thyroid follicles but have a defect somewhere in the pathway of thyroid hormone synthesis. To understand the molecular mechanism underlying this phenotype, positional cloning of *kmi* is now underway.

kmi is a hypothyroid mutant

To determine whether *kmi* mutants can secrete thyroid hormones, we performed a radioimmunoassay (RIA) to measure the content of total plasma T4 and 3,5,3'-triiodothyronin (T3) (Fig. 2). The RIA revealed that *kmi* mutants are able to secrete thyroid hormones. However, the

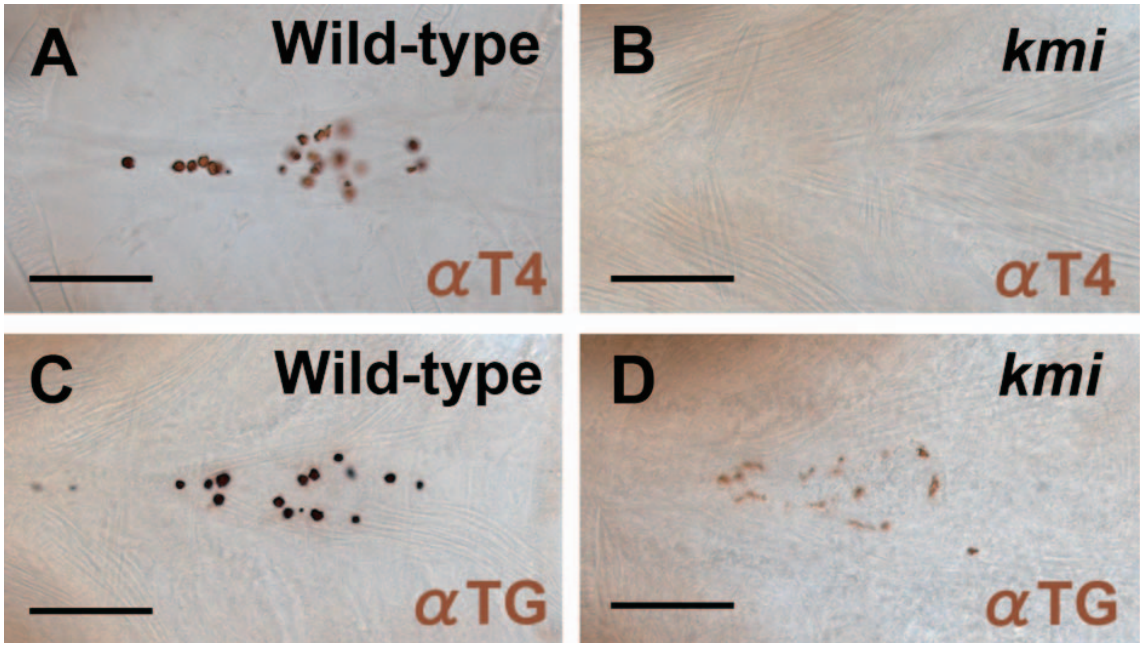

Fig. 1. Immunostaining of T4 and TG in wild type and *kmi*. Ventral views, anterior to the left; all larvae are at hatching stage. T4 immunoreactivity was undetectable and TG immunostaining was very weak in the *kmi* mutant. **(A, C)** Thyroxine (T4) and thyroglobulin (TG) immunostain the thyroid follicles of wild-type larvae in A and C, respectively. **(B, D)** T4 immunoreactivity was undetectable in *kmi* mutant larvae. TG immunostaining was very weak in the thyroid follicles, which are small and irregularly shaped. Scale bar, 100μm.

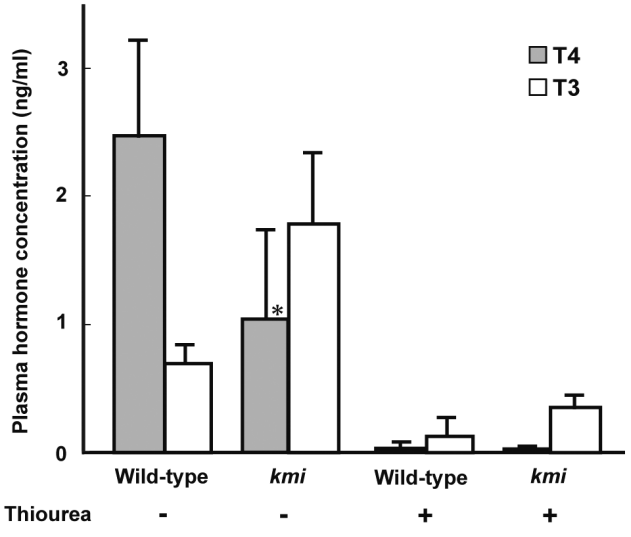

Fig. 2. Radioimmunoassay of T4 and T3 in wild type and *kmi* with or without thiourea (TU) treatment. The assay revealed low plasma T4 but high T3 levels in the *kmi* mutant. Plasma samples were taken from five mating pairs each (n=10) for T4 and T3. The mean T4 level of wild type was significantly greater than that of *kmi* mutants (* Mann-Whitney *U*-test, $P<0.05$). In contrast, the mean T3 level of *kmi* mutants was greater than that of wild type. TU treatment (0.003%, 10 days) significantly reduced the levels of both T4 and T3, but a substantial amount of T3 remained in both wild type and *kmi*.

mean content of plasma T4 in *kmi* was significantly reduced to only 41% of wild type ($P<0.05$, *U*-test). Interestingly, the concentration of plasma T3 increased to 257% that of wild type. Parallel results were observed in *kmi* after TU treatment (Fig. 2). T3, which is an active form of thyroid

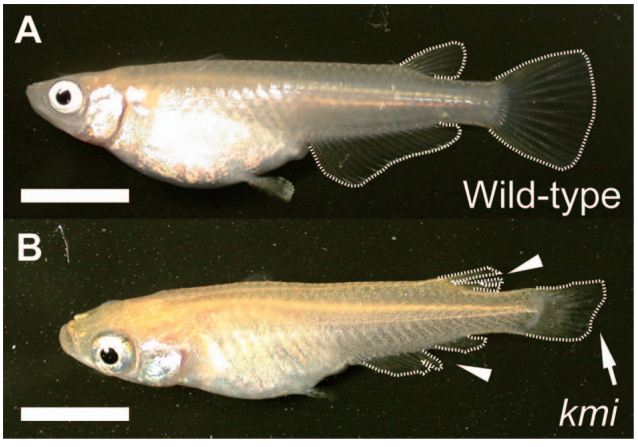

Fig. 3. Appearance (lateral views) of adult female wild-type and *kmi* medaka. **(A)** Wild-type adults rarely showed injury in fins. **(B)** *kmi* mutants often displayed ragged, damaged fins. Arrowheads indicate slits in the dorsal and anal fins; arrow indicates the jagged distal margin of the caudal fin; dotted lines demarcate the outlines of the dorsal, anal, and caudal fins. Scale bar, 5 mm.

hormone, is known to be generated mainly from T4 by deiodinase at target tissues. At the moment, we do not know the reason for this increase in T3, but in *kmi,* the conversion of T4 into T3 may be upregulated. The lower plasma content of T4 can be ascribed to its lower secretion rate in mutant follicular cells.

Hypothyroidism delays fin regeneration in medaka fish

As described above, *kmi* mutants often show damaged fins, which are never seen in wild-type medaka under nor-

mal laboratory conditions (Fig. 3). This can be explained by defective fin regeneration in the mutant. To test this idea, we performed a caudal fin amputation experiment in which we measured the lengths of regenerates between 0 and 14 days postamputation (dpa). Fig. 4C clearly shows that the regenerative length of *kmi* at 14 dpa was significantly shorter ($P<0.05$, *t*-test), about 93% of wild type. Thus, the *kmi* mutation delays the regeneration process of the caudal fin in medaka.

To directly examine the relationship between regeneration delay and thyroid hormone, we treated fish with thiourea (0.003%), a goitrogen that blocks iodination of thyroglobulin, from the day of fin amputation. This treatment increased the difference in growth between wild-type and mutant regenerates; the regenerative length of *kmi* was significantly shorter ($P<0.01$, *t*-test; Fig. 4C), only about 70% that of wild type. This result can be explained if we assume that wild-type medaka are more resistant to thiourea treatment because they retain some amount of T4 in the colloidal lumen of the follicle. Indeed, as described above, the mutant follicles did

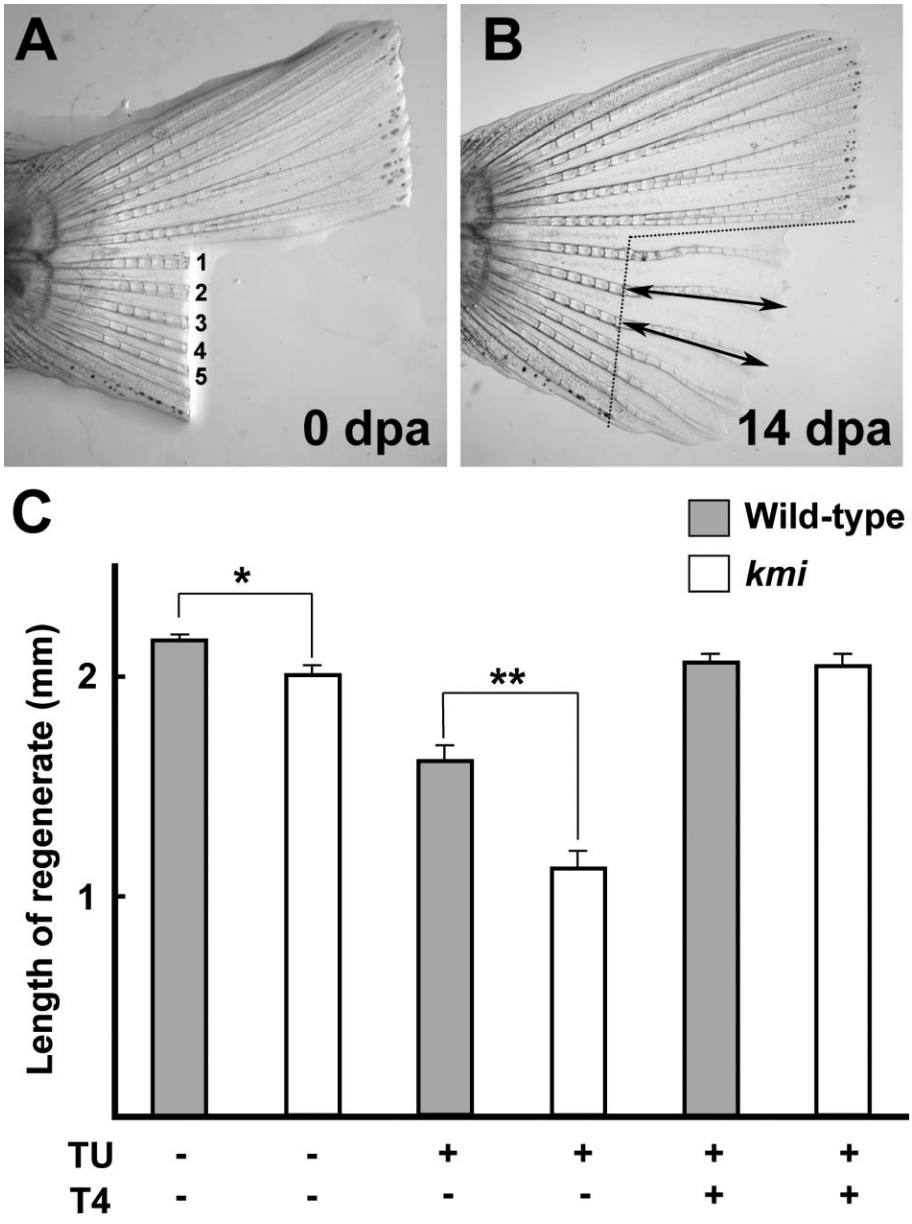

Fig. 4. Amputated medaka caudal fins and their regeneration. **(A, B)** Amputated caudal fins of wild-type adult female fish at 0 and 14 days post amputation (dpa), respectively. Bony rays are numbered from median to ventral. Arrows indicate the regenerative length of rays 2 and 3. 'Length of regenerate' for each individual was defined as the average of regenerated lengths of rays 2 and 3. The dotted line in B demarcates the outline of the regenerated fin at 14 dpa. **(C)** Length of caudal fin regenerate. The regenerative length of *kmi* was only 93% that of wild type in the TU-T4- group (17 mating pairs each; *$P<0.05$, *t*-test). The difference in length was enhanced in the TU+T4- group (eight mating pairs each; **$P<0.01$, *t*-test); the regenerative length of *kmi* was only 70% that of wild type. There was no significant difference between *kmi* and wild type in the TU+T4+ group (eight mating pairs each). Both TU (0.003%) and L-thyroxine (T4, 30 ng/ml) treatments started from the day of amputation.

not show any trace of T4 immunoreactivity in their lumens (Fig. 1B). Importantly, when T4 (30 ng/ml) was administered in addition to thiourea from 0 dpa, the regenerative length was restored in both wild type and *kmi,* and no significant difference was detected between them (Fig. 4C). These results indicate that thyroid hormone is required for normal fin regeneration in medaka.

Hypothyroidism affects the rate of fin regeneration

We then examined in detail the process of fin regeneration under normal and hypothyroid conditions. The fish groups examined were as follows. (1) Control group: no pharmacological treatment. (2) Short-TU group: thiourea (0.003%) treatment from the day of fin amputation. (3) Long-TU group: thiourea treatment from 20 days before and after the day of fin amputation. (4) Short-TU+T4 group: thiourea (0.003%) with T4 (30 ng/ml) treatment from the day of fin amputation. To calculate growth rates, regenerating fins were measured every other day for two weeks.

As represented by the control group (Fig. 5B), the normal growth rate did not remain constant throughout the regeneration period: it slowed at 2 dpa (0.08 mm/day), peaked at 6 dpa (0.26 mm/day), and thereafter gradually decreased. As expected, the long-TU group showed the lowest growth rate throughout the entire period (except for day 10). The difference in growth rate ultimately resulted in a significant difference in regenerative length between the control and long-TU groups (Fig. 5A; $P<0.01$, *t*-test). The Short-TU group, on the other hand, showed the same growth rate as the control group for the first 4 days, but its growth rate gradually slowed and became as low as the long-TU group by day 10. In the short-TU+T4 group, the growth rate was slightly lower for the first 6 days, but finally caught up with the normal rate at day 8.

It was previously shown that after thiourea treatment in medaka, the plasma T4 concentration gradually falls below the detection limit in a week (Tagawa and Hirano, 1991). Indeed, we confirmed that the plasma levels of both T4 and T3 were much lower, 1% and 16% respectively, at 10 days of TU treatment (Fig. 2). This is consistent with our observation that a difference in growth rate between the control and short-TU groups becomes evident after 6 dpa (Fig. 5B).

The role of thyroid hormone in fin regeneration

Teleost fin regeneration consists of at least three stages (Poss *et al*., 2003): wound healing, blastema formation (a blastema is a mass of undifferentiated and proliferative cells underneath the epidermis covering a wound), and regenerative outgrowth. Katogi *et al.* (2004) histologically examined the regenerative process of amputated caudal fins in medaka and reported that at 28°C wound healing and blastema formation were completed by 1 dpa and 3 dpa, respectively. Thus, the growth rate after day 4 in Fig. 5B represents mainly the rate of regenerative outgrowth. Our data thus demonstrate that thyroid hormone positively regulates regenerative outgrowth.

Furthermore, thyroid hormone is known to promote the rate of wound healing in mammals (Lennox and Johnston, 1976; Safer *et al*., 2004, 2005). Hypothyroidism could similarly affect the process of wound healing in fish. This is consistent with the result that the growth rate of the long-TU

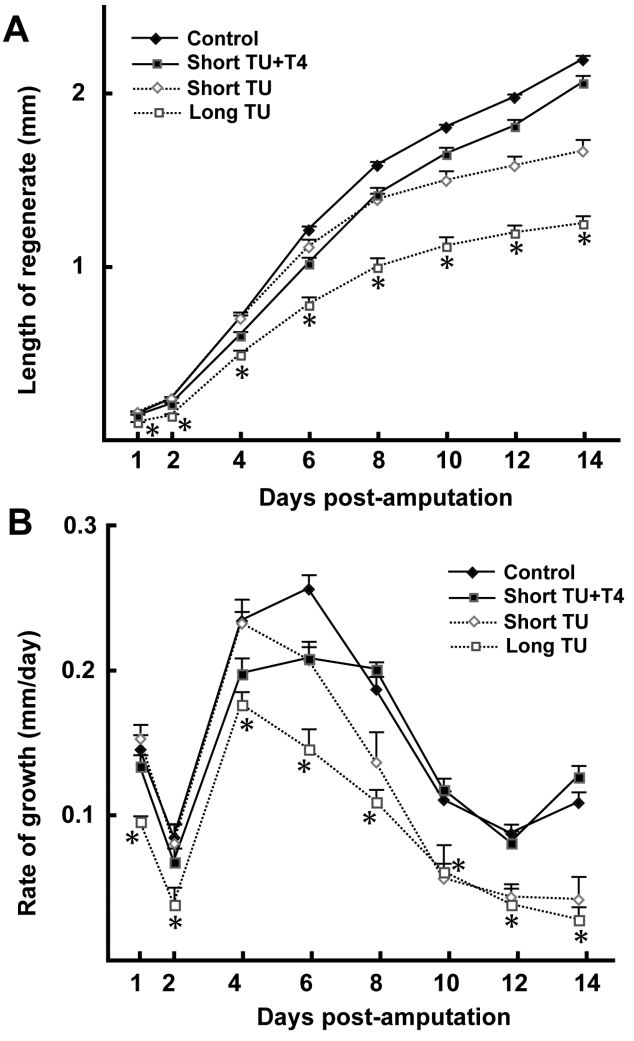

Fig. 5. Hypothyroidism decreases the regeneration rate of amputated caudal fins. Control: no pharmacological treatment; 10 mating pairs (n=20) were used. Short TU: thiourea (0.003%) treatment from the day of fin amputation; five mating pairs (n=10) were used. Long TU: thiourea treatment from 20 days before and after the day of fin amputation; seven mating pairs (n=14) were used. Short TU+T4: thiourea (0.003%) and T4 (30 ng/ml) treatment from the day of fin amputation; eight mating pairs (n=16) were used. In the short-TU group, two males died by accident, on days 2 and 10, and data for a female fish on day 14 were removed because of severe injury to the regenerate. **(A)** Length of regenerate under various T4 conditions. **(B)** Fin regeneration rates under various T4 conditions. The growth rate of the long-TU group was lowest among the experimental groups throughout the regeneration period (except for day 10). The growth rate of the short-TU group resembled that of the control group for the first 4 days, but decreased to the level of the long-TU group from around day 8 onwards; * means of the long-TU and control groups were significantly different ($P<0.01$, *t*-test).

group was significantly lower than that of the control group from the earliest time point, day 1 (Fig. 5B; $P<0.01$, *t*-test), although longer and earlier treatment of TU could have some side effects other than hypothyroidism.

While performing the above experiments, we noticed that the growing regenerates of the short- and long-TU groups were fragile and easily damaged (Fig. 6D). In con-

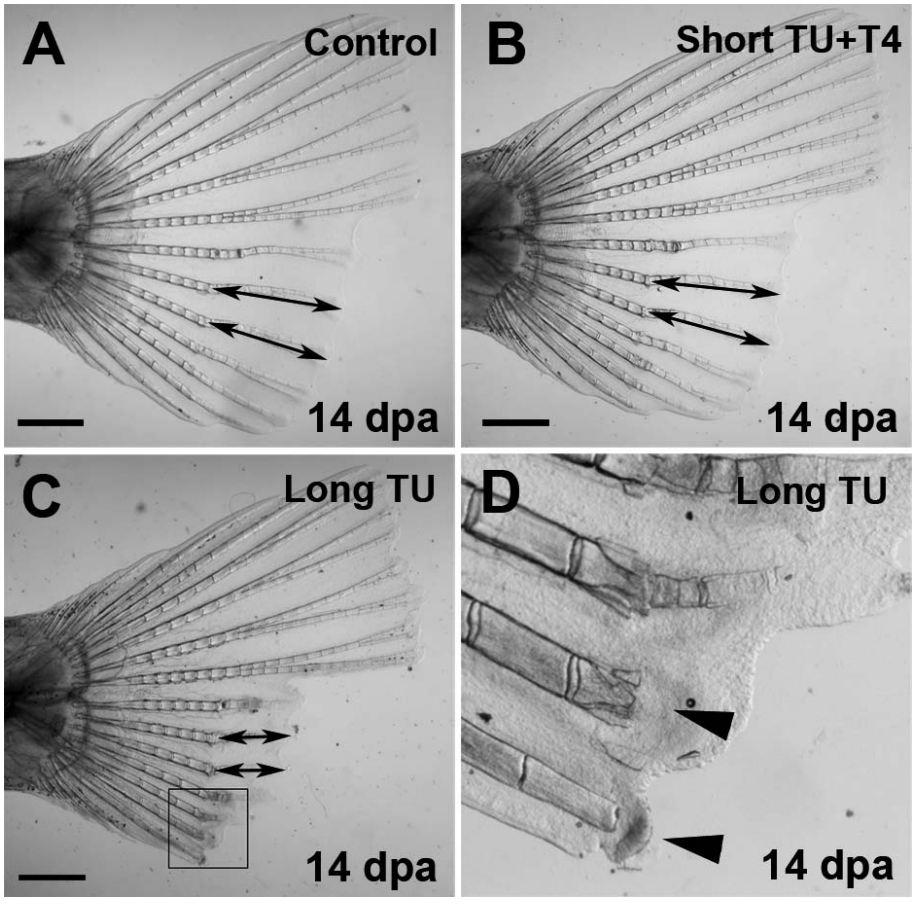

Fig. 6. Representative appearance of regenerating fins at day 14. **(A)** Regenerating caudal fin of the control group. **(B)** Regenerating caudal fin of the short-TU+T4 group was slightly shorter than that of the control group, but appeared to regenerate normally. **(C)** The long-TU group showed severely retarded fin regeneration. **(D)** High-magnification image of the box in (C). Arrowheads indicate severely damaged fin rays in the regenerate. It seems that these fin rays were formed initially but became partially missing or disorganized during regeneration, probably due to lack of strength of the regenerate. Scale bar, 1 mm.

trast, this never happened in the fin regenerates of the control or short-TU+T4 groups during the experiments. This suggests that hypothyroidism affects cell-differentiation and/or proliferation processes that ensure the mechanical strength of regenerates, especially in the epidermis. This is again consistent with the observation that in wound healing, exogenous T4 improved the strength of the scars, partially through positive regulation of *keratin* gene expression (Lennox and Johnston, 1973; Safer *et al*., 2004, 2005). Taken together, thyroid hormone could be involved in nearly all stages of caudal fin regeneration.

Conclusion

The present study demonstrates for the first time the direct involvement of thyroid hormone in teleost fin regeneration. The first indication of this came from the curious phenotype of the medaka *kmi* mutant, and we then systematically examined the role of thyroid hormone using medaka with pharmacologically induced hypothyroidism. This experiment has provided reliable data confirming that the medaka is a good model system for regeneration study. With the medaka draft genome (Naruse *et al*, 2004; http://medaka.utgenome.org/) and cDNA microarrays (Kimura *et al*, 2004; http://medaka.lab.nig.ac.jp/) available, a future intensive search for thyroid-hormone response genes involved in fin regeneration will give us a new understanding of the role of thyroid hormone in epimorphic regeneration.

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